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Establishment of a rapid drug screening system based on embryonic stem cells

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ABSTRACT

Embryonic stem (ES) cells have the capacity for self-renewal and differentiation into three germ layers following formation of embryonic bodies (EB). To investigate toxicity of pharmaceutical compounds, five toxic chemicals, indomethacin, dexamethasone, hydroxyurea, 5-fluorouracil, and cytosine arabinoside were applied in mouse ES cells during formation of EBs. Using microscopic evaluation, the size of EBs was reduced in a dose-dependent manner by treatment with pharmaceutical chemicals. While apoptosis-related proteins, cleaved caspase-3 and PARP, were decreased in compound-exposed EBs, necrosis-related protein (Hmgb1) was present in culture media of EBs, indicating that detection of Hmgb1 can result in activation of necrosis by pharmaceutical compounds. While pharmaceutical compounds impaired the differentiation of mES cells linked with spontaneous apoptotic cell death, it was determined that cytotoxic cell damage is necrosis-dependent in mES cells. In addition, an apoptotic transcript (Noxa mRNA) in toxicant-exposed EBs was decreased in parallel with apoptosis-related proteins. Following impairment of apoptosis, differentiation-related markers including un-differentiation (*Sox2*), endoderm (*Hnf4*), mesoderm (*Bmp4*), and ectoderm (*Pax6*) also fluctuated by treatment with pharmaceutical compounds. Taken together, the data imply that exposure to pharmaceutical compounds results in increased cell death hindering the spontaneous apoptosis of cells to undergo differentiation. Using both characteristics of ES cells like self-renewal or cellular pluripotency and potentials of ES cells for evaluation in toxicity of various compounds, the current study was conducted for establishment of a novel drug screening system beyond hidden virtues of the well-known chemicals.

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1. Introduction

Toxic chemicals are generally referred to as substances that damage an organism and animal or mammalian cells; these substances have been used in an attempt to detect toxicants *in vivo* or *in vitro*, respectively. Animal testing provides more precise physiological conditions for evaluation of toxic chemicals, however, it also has some disadvantages, such as economic and ethical constraints (Hofer et al., 2004). Many toxicologists, as well as our group, have focused on the *in vitro* toxicity testing method without ethical issues regarding experimental animals. This was related to prohibition of animal testing for cosmetic products after 2007 by the European Union. As a result, cosmetic companies focused on alternative testing to substitute for experimental animals. For evaluation of new compounds or drugs before preclinical or clinical trial, establishment of efficient drug screening is needed as a time or cost saving method because most of them were dropped out in clinical trial because of side effects or insufficient effects (Prowell, 2014). It is likely that the cost of drug development has increased by three times that of the 1990s because many countries have reinforced safety testing for new drug or compounds. Since expiration of the patent of blockbuster drugs, major pharmaceutical companies have diminished funds for investment in development of new drugs. Otherwise, some pharmaceutical companies focused on the drug repositioning of well-known compounds (Chong and Sullivan, 2007). There is some evidence for drug repositioning. Although VIAGRA® was developed and used in treatment of patients with angina, it has been used as an agent for treatment of erection impotency (Cheitlin et al., 1999). As an agent for diabetes mellitus, metformin is currently used as an anticancer agent (Franciosi et al., 2013). In addition, salinomycin for lepers was used as an anticancer agent for various cancers (Huczynski, 2012).

In the field of developmental toxicology, embryonic stem (ES) cells have been used for testing of pharmaceutical chemicals due to their potential for differentiation into three germ layers, endoderm, mesoderm, and ectoderm (Yamanaka et al., 2008). For evaluation of developmental toxicity of compounds, an embryonic stem cell test was introduced by the European Center for the Validation of Alternative Methods (Genschow et al., 2004); it is assessed by formation of embryonic bodies (EBs) indicating the onset of differentiation of ES cells during early embryogenesis. Based on the EST method, many research groups have refined the developmental toxicity of a variety of compounds (de Jong et al., 2009; Eckardt and Stahlmann, 2010). Toxic chemicals were also examined in the ES-EB system using the inhibition of cytotoxicity (IC_{50}) and inhibition of differentiation (ID_{50}) values, and were then divided into three major classes, non-embryotoxic, weakly embryotoxic, and strongly embryotoxic (Genschow et al., 2000, 2004). After the first description of mouse ES cells from pre-implantation embryo, it is now known that these cells have novel characteristics, including self-renewal and cellular pluripotency. According to a recent report (Chambers, 2004), the self-renewal is related to patterns of gene expression, such as *Oct4* or *Nanog* involved with transcriptional programming. Using mES cells, we evaluated the effects of well-known developmental toxic chemicals, including indomethacin, dexamethasone, hydroxyurea, 5-fluorouracil, and cytosine arabinoside. Despite extensive use of these chemicals as anticancer and anti-inflammatory agents, indomethacin (Norton, 1997), dexamethasone (Xu et al., 1994), hydroxyurea (Hansen et al., 1995), 5-fluorouracil (Lau et al., 2001), and cytosine arabinoside (Ninomiya et al., 1994), respectively, have been shown to be developmentally toxic, causing disrupted development of the embryo due to interference in DNA synthesis or protein production. This study was conducted in order to investigate the inhibition of differentiation by toxic chemicals using alternative *in vitro* models, and evaluated the expression of marker genes related to formation of EBs or the cell death signal, which may play important roles in reduction of EBs. Following characteristics of stem cells for tissue regeneration or homeostasis of the body, this study will be useful in establishing a novel drug screening system for drug candidates beyond drug repositioning, hidden virtues of the well-known chemicals.

2. Material and methods

2.1. Chemicals and antibodies

Indomethacin, dexamethasone, hydroxyurea, 5-fluorouracil, and cytosine arabinoside were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bax (sc-7480), Gapdh (sc-20357) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hmgb1 (#3935) and Cleaved Caspase Antibody Sampler Kit (#9929) including cleaved Caspase-3 and PARP were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. ES cell culture and treatment

Mouse embryonic stem (mES) cells were purchased from the American Type Culture Collection (ES-E14TG2a cell line; Manassas, VA, USA). ES cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture DMEM/F-12 (GIBCO, Grand Island, NY, USA) supplemented with nonessential amino acids, 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA), 2-mercaptoethanol (2-ME; 10^{-4} M), penicillin (100 U/ml), and streptomycin (100 μg/ml). The mouse embryonic fibroblasts (mEFs) were obtained from E12.5 ICR mouse embryos that had been mitotic inactivated with 10 μg/ml mitomycin C (Sigma-Aldrich) for 3 h, plated at a density of 1.6×10^6 cells/well on 6-well plates (Nunclon™ Surface; NUNC, Wiesbaden, Germany), and cultured overnight. The cells were cultured at 37 °C in a 5% CO_2 humidified tissue culture incubator (SANYO, San Diego, CA, USA).

Undifferentiated cells were maintained with leukemia inhibitory factor (LIF; 10^3 U/ml; Esgro, Chemicon, Temecula, CA) on a feeder layer of mEFs. To induce differentiation, mES cells were suspended in DMEM/F-12 containing non-essential amino acids, 15% FBS, 2-ME (10^{-4} M), penicillin (100 U/ml), and streptomycin (100 μg/ml) without LIF on mitomycin C-treated mEFs for 2 days. To induce EBs formation using the hanging-drop culture method, the mES cells were cultured with density of 800 numbers per 25 μl drop in media including indomethacin, dexamethasone, hydroxyurea, 5-fluorouracil,